Conjugative plasmid transfer between *Salmonella enterica* Newport and *Escherichia coli* within the gastrointestinal tract of the lesser mealworm beetle, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)[1]

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ABSTRACT The objective of this study was to determine if conjugative transfer of antimicrobial resistance plasmids could occur between donor and recipient bacteria within the gastrointestinal tract of lesser mealworm beetles, a common pest in poultry production facilities. In 3 replicate studies (n = 40 overall), beetles were allowed to feed for 2 h on brain heart infusion agar inoculated with a multidrug-resistant *Salmonella enterica* serotype Newport strain (SN11 that carried plasmid replicons A/C and N) at 1.0×10^8 cfu/mL. Beetles were surface-disinfected and allowed to feed for 16 h on brain heart infusion agar inoculated with nalidixic acid- and rifampicin-resistant *Escherichia coli* JM109 at 9.0×10^6 cfu/mL. After bacterial exposure, beetles were surface-disinfected, homogenized, and selectively plated for transconjugants. Serial dilutions were done for conjugation frequencies. In vitro filter conjugations were performed simultaneously with beetle conjugations. Transconjugants were produced in all beetles exposed to both donor and recipient bacteria. Ninety-five percent of the beetle and 100% of the in vitro filter transconjugants were positive for the N plasmid replicon. The A/C replicon, which was also detected in the SN11 donor strain, did not transfer in any of the conjugation studies. None of the transconjugants displayed resistance to extended-spectrum cephalosporins. The geometric mean conjugation frequency in the beetle gut was 1.07×10^{-1}. The average conjugation frequencies for the beetle gut were 2 logs higher than those for the filter conjugations 4.1×10^{-3}. This study demonstrates that horizontal transfer of antimicrobial resistance plasmids can occur between *Salmonella* and *E. coli* within the gut of beetles and that beetles may be used as an in vivo model to study resistance gene transfer.

Key words: darkling beetle, *Alphitobius diaperinus*, antimicrobial resistance, poultry litter

2009 Poultry Science 88:1553–1558
doi:10.3382/ps.2008-00553

INTRODUCTION

The emergence of multidrug-resistant (**MDR**) foodborne pathogens such as *Salmonella* and *Escherichia coli* has become a global public health concern. In large-scale poultry and livestock production facilities, antimicrobial agents may be used for therapeutic, prophylactic, or subtherapeutic growth promotion purposes (Baquero et al., 1997; Gustafson and Bowen, 1997). It is well known that such use of antimicrobial agents puts selective pressure on commensal and pathogenic bacteria contributing to the clonal expansion of MDR strains (Baquero et al., 1997). Because MDR bacteria may persist in some environments, there is a need to understand the ecology of these strains and their resistance determinants. This includes potential reservoirs for bacteria as well as sites where transfer of mobile resistance elements, such as plasmids, could occur between commensal and pathogenic bacteria.

Insects that inhabit the poultry litter and underlying soil are a potential reservoir for MDR bacteria (McAllister et al., 1994, 1996). One of the most common insects present in poultry litter is the lesser mealworm, *Alphitobius diaperinus* (Panzer), formerly called the darkling beetle (Axtell and Arends, 1990; Rueda and Axtell, 1997; Axtell, 1999). All life stages of this insect can be found inhabiting and feeding within the litter (Axtell and Arends, 1990; Axtell, 1999). These insects are omnivorous scavengers that feed on fecal material, spilled chicken feed, cracked eggs, chicken carcasses, house fly maggots, and detritus (Axtell, 1999). In turn, they are often eaten by chickens, wild birds, and rodents within the poultry house (Axtell and Arends, 1990). Because lesser mealworm beetles are extremely long-lived and are able to endure adverse conditions, they are difficult to control within poultry houses and often persist be-

Received December 16, 2008.
Accepted April 20, 2009.
[1]Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product, or exclusion of others that may be suitable.
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tween flock rotations (Axtell, 1999; Crippen and Sheffield, 2006). Tilling the litter and adding fresh litter between flock rotations aides in supporting beetle infestations. In northern climates, freezing temperatures aid in controlling beetle infestations; however, southern climates do not have this advantage.

Beetles have also been shown to harbor several viral, bacterial, and fungal pathogens that affect bird health and performance (De las Casas et al., 1968, 1972, 1973, 1976; McAllister et al., 1994, 1995, 1996). These factors make them potential participants in the dissemination of pathogenic bacteria within the poultry house environment. The harboring of bacteria internally, not just externally by these pests within production facilities, further complicates the dilemma facing producers to limit the spread of infectious pathogens. Crippen and Sheffield (2006) developed an external disinfection method that allowed the study of bacteria that were present in the gastrointestinal tract of the lesser mealworm beetles. A subsequent study demonstrated that beetles are capable of rapid oral consumption of *Salmonella* from relatively low levels of environmental contamination given favorable conditions (Crippen et al., 2009).

Studies have shown that horizontal transfer between donor and recipient bacteria can occur within insects (Armstrong et al., 1990; Axtell, 1999; Petridis et al., 2006). However, none to date have investigated conjugation in the gut of the lesser mealworm beetle. The purpose of this study was to determine if horizontal transfer of antimicrobial resistance plasmids, via conjugation, could occur between *Salmonella* (donor) and *E. coli* (recipient) within the gastrointestinal tract of lesser mealworm beetles.

MATERIALS AND METHODS

Beetles

The colony of *A. diaperinus* that was started at the USDA, Agricultural Research Service, Southern Plains Agricultural Research Center has been described previously (Crippen and Sheffield, 2006). Beetles were reared in 1,000 mL of wheat bran (Morrison Milling Co., Denton, TX) in plastic containers (15 × 15 × 30 cm) with screen tops and held at 30°C in an 8L:16D cycle. Each cage contained a 6-cm^2 sponge moistened with deionized water and a 0.5-cm-thick slice of a medium-sized apple replenished twice per week, and 30 mL of fishmeal (Omega Protein Inc., Hammond, LA) was added to the wheat bran once per week.

Bacterial Isolates

Salmonella enterica serotype Newport (donor) isolate (SN11) is positive for the plasmid replicons N and A/C by PCR-based replicon typing (**PBRT**) and is resistant to amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline. *Escherichia coli* strain JM109 (recipient) was made resistant to nalidixic acid and rifampicin and was found to be an efficient recipient for SN11. It has been found that JM109 is positive for chromosomal replicons FIA, FIB, and FIC. Overnight cultures were grown in brain heart infusion broth (**BHIB**), centrifuged, washed, resuspended in PBS, and adjusted to approximately 10^7 cfu/mL by spectrophotometry at optical density at 600 nm = 0.6 to 0.7 for subsequent conjugation experiments.

Beetle Conjugation Experimental Design

Three replications of each experiment were done using at least 10 beetles per group. Exposure tubes consisted of 7.0 mL of brain heart infusion agar (**BHIA**; Difco, Detroit, MI) in a 17 × 100 mm (14 mL) sterile tube inoculated with 10.0 μL of 1) PBS, 2) donor SN11 at a mean of $1.4 \times 10^8 \pm 9.4 \times 10^7$ cfu/mL, or 3) recipient JM109 at a mean of $9.3 \times 10^6 \pm 7.7 \times 10^6$ cfu/mL. Exposure tubes were incubated for 1 h at 37°C to produce a lawn of bacteria on the agar surface before the addition of beetles. Two beetles 4 to 5 wk of age were added per tube and were allowed to move freely at 30°C in the dark. It has been previously shown that beetles eat the agar and bacteria in the exposure tubes (Crippen and Sheffield, 2006; Crippen et al., 2009). The experimental groups consisted of group 1) beetles placed on PBS tubes for 18 h, group 2) beetles placed on SN11 tubes for 2 h and PBS for 16 h, group 3) beetles placed on PBS for 2 h then JM109 tubes for 16 h, and group 4) beetles placed on SN11 tubes for 2 h and JM109 tubes for 16 h. At the end of all 2-h incubations, beetles were surface-disinfected by a 2-min immersion in SporGon (Decon Labs Inc., Bryn Mawr, PA), followed by transfer to an empty containment tube for 1 to 2 min, and were then placed into the appropriate secondary exposure tube for the 16-h incubation. After all 16-h incubations, beetles were surface-disinfected using a sequential immersion in 95% ethanol followed by SporGon, as described previously (Crippen and Sheffield, 2006). After all disinfection procedures, beetles were immersed in BHIB and the broth was incubated at 37°C overnight and subcultured onto BHIA to detect surface contamination. Any beetle found to be external contamination post-surface disinfection was removed from the study. After the 16-h incubation and disinfection, beetles were placed individually into 1.5 mL of BHIB, homogenized, and the homogenates were plated on selective media. For calculation of conjugation frequencies, homogenized beetles were serially diluted in PBS and plated.

Selective Isolation

One hundred microliters of the beetle homogenates from groups 1, 2, and 3 were plated in duplicate onto MacConkey agar (Becton Dickinson, Sparks MD) supplemented with 32 μg/mL each of nalidixic acid and rifampicin (**MAC-NR**), supplemented with 32 μg/mL

of tetracycline (**MAC-T**), and supplemented with 32 μg/mL of nalidixic acid, rifampicin, and tetracycline (**MAC-NRT**). Group 4 homogenates were plated in quadruplicate onto MAC-NRT.

Isolated colonies were selected from MAC-T and MAC-NR plates from groups 2 and 3, respectively, for subsequent validation as SN11 and JM109. Isolated colonies from each of the 4 MAC-NRT plates in group 4 were also collected for subsequent validation as putative transconjugants. Each colony was subcultured onto BHIA and incubated at 37°C for 16 h and subcultured a second time onto trypticase soy agar with 5% sheep blood (TS-blood agar; BVA Scientific, San Antonio, TX) and BHIA. The bacteria from these isolation plates were used for indole production testing, antimicrobial susceptibility testing, and molecular characterization of plasmid replicons.

Filter Conjugation

In vitro filter conjugations were done simultaneously with each replicate beetle study using the same overnight donor and recipient preparations. The SN11 and JM109 conjugations were done on solid filter supports at a 1:10 ratio (donor:recipient).

The JM109 cells fed to the beetles or collected on membrane filters were quantitated by serial dilution and plating onto MAC-NR and MAC-NRT media, respectively. The plates were incubated overnight at 37°C. The conjugation frequency of plasmids from donor to recipient cells was calculated as the ratio of the number of transconjugants from MAC-NRT to the number of recipients on MAC-NR. This was determined both in vivo in the beetle gut and in vitro by filter-mating and was repeated in the 3 different experiments to establish a mean.

Transconjugant Characterization

All putative transconjugants isolated for further characterization were tested for indole production using 1% p-dimethylaminocinnamaldehyde (Indole Reagent, Anaerobe Systems, Morgan Hill, CA) per the instructions of the manufacturer. *Escherichia coli* cleaves indole from tryptophan producing a blue color reaction. Only indole-positive isolates were further characterized as putative transconjugants.

The antimicrobial minimum inhibitory concentration for growth was determined by broth microdilution according to the Clinical Laboratory Standards Institute (**CLSI**; CLSI, 2003). Antimicrobial susceptibility of bacteria from the trypticase soy-blood agar isolation plate for each beetle was performed using a Sensititer automated antimicrobial susceptibility system according to the instructions of the manufacturer (Trek Diagnostic Systems, Cleveland, OH) and a National Antimicrobial Resistance Monitoring System panel for gram-negative enteric bacteria (CMV1AGNF, Trek Diagnostic Systems). The following antimicrobials were evaluated on the CMV1AGNF 96-well plate: amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. Rifampicin (Sigma-Aldrich Co., St Louis, MO) susceptibility was determined manually by broth microdilution using the methods described by the CLSI (2003). The following American Type Culture Collection (Manassas, VA) strains were used as controls for antimicrobial susceptibility testing: *E. coli* 25922, *Enterococcus faecalis* 29212, *Staphylococcus aureus* 29213, and *Pseudomonas aeruginosa* 27853. Data were interpreted using CLSI breakpoints (CLSI, 2005).

PBRT

The method used for replicon typing has been described previously (Carattoli et al., 2005). In this study, PBRT was done on SN11 and the *E. coli* transconjugants. The replicon types tested included: B/O, K, FIA_S, FIA, FIB, FIC, HI1, HI2, Y, I1, repF, X, L/M, N, P, W, T, and A/C. Positive controls for these replicons were provided by Istituto Superiore di Sanità (Rome, Italy). Transconjugants were tested for all replicons from in vivo conjugations to determine if they acquired any plasmid replicons from the normal flora of the beetle gut.

RESULTS

Beetle and Filter Conjugations

One hundred percent (n = 40) of the beetles from group 4 produced transconjugants on MAC-NRT (Table 1). As expected, no growth of SN11 occurred on MAC-NR or MAC-NRT and no growth of JM109 occurred on MAC-T or MAC-NRT (Table 1). On the MAC-T plates from groups 1 and 3, there were 5% (n = 4 plates) and 25% (n = 20 plates) that were positive for growth, respectively (Table 1). On MAC-NRT from group 3, there was 6.25% (n = 10 plates) growth. Isolates from these plates were further characterized and were not SN11 or JM109, and were therefore considered to be normal flora from the beetle gut.

The results of the filter conjugations are shown in Table 1. One hundred percent of the filter conjugations from group 4 produced putative transconjugants. Because these conjugations were done with pure cultures, no contaminating microflora were observed.

Transconjugant Characterization

Four putative transconjugants isolated from each of the 40 beetle homogenates were characterized. All 160 selected isolates were indole-positive, indicating they were likely *E. coli*- and not *Salmonella*-possessing mutations conferring nalidixic acid and rifampicin resistance. Eighty-eight percent (n = 141) of the 160 putative transconjugants displayed resistance to ampicillin,

Table 1. Beetle and filter conjugation results from selective plating[1]

Item	No. of beetles	MAC-T (n = 80)	MAC-NR (n = 80)	MAC-NRT (n = 160)
		% of plates with growth		
Beetle group[2]				
1	40	5	0	0
2	40	100	0	0
3	40	25	80	6.25
4	40	ND[3]	ND	100
Filter group	No. of filters	MAC-T (n = 18)	MAC-NR (n = 18)	MAC-NRT (n = 36)
1	9	0	0	0
2	9	100	0	0
3	9	0	100	0
4	9	ND	ND	100

[1]MAC-T = MacConkey agar supplemented with 32 μg/mL of tetracycline; MAC-NR = MacConkey agar supplemented with 32 μg/mL each of nalidixic acid and rifampicin; MAC-NRT = MacConkey agar supplemented with 32 μg/mL of nalidixic acid, rifampicin, and tetracycline.

[2]Group 1 = PBS control; 2 = donor only, SN11; 3 = recipient only, JM109; 4 = putative transconjugant.

[3]ND = not done.

chloramphenicol, nalidixic acid, rifampicin, streptomycin, sulfisoxazole, and tetracycline, but not to the extended-spectrum cephalosporins (Table 2). The same phenotype was displayed by 100% (n = 12) of the filter group 4 transconjugants in the 3 replicate experiments. Two and 14 of the putative beetle transconjugants displayed resistance to ceftiofur and cefoxitin, respectively, but none of the other cephalosporins. The phenotypic resistance to 9 antimicrobial agents displayed by SN11 was detected in only 2 beetle transconjugants. However, the A/C replicon that carries the cephalosporin resistance gene bla_{CMY} was not detected in these 2 isolates. All beetle and filter transconjugants were resistant to both nalidixic acid and rifampicin, suggesting they were the experimental *E. coli* JM109 and not other lactose-fermenting gram-negative strains from the normal flora of the beetle gut.

All putative transconjugants were tested by PBRT. This revealed that 100% of the transconjugants from the filter group 4 were positive for replicons N, FIA, FIB, and FIC, but not A/C (Table 2). No other plasmid replicons from the PBRT panel were detected. The presence of FIA, FIB, and FIC indicates that transconjugant colonies were the JM109 recipient strain. Of the putative transconjugants selected from the beetle (group 4) conjugations, 95% (n = 152) possessed the same replicon profile as the transconjugants produced by filter conjugation (Table 2). There was some variation in the detection of FIA, FIB, and FIC, but only 1 of the 160 transconjugants was negative for the N replicon and all other replicons tested. This isolate was MDR and may have had a mutation on the N plasmid that prevented amplification of N by PCR. Overall, these data showed that replicon N but not A/C was transferred from SN11 to JM109.

Transconjugant Frequency

Filter and beetle replicate studies were done using the same SN11 and JM109 preparations to confirm the conjugative ability of the donor and recipient preparations. The average geometric mean conjugation frequencies were 1 to 2 logs higher for conjugations done in the beetle gut as compared with filters (Table 3).

DISCUSSION

Poultry producers have long been under pressure to limit the dissemination of infectious pathogens that are often present on retail products (Zhao et al., 2001, 2006). However, with the emergence of MDR pathogens, there are new pressures to limit the use of antimicrobial agents. This poses an additional dilemma for producers with regard to the maintenance of healthy

Table 2. Phenotypic and genotypic profiles used for characterization of *Salmonella* Newport SN11 (donor), *Escherichia coli* JM109 (recipient), and *E. coli* transconjugant bacteria[1]

Isolates	SN11 donor	JM109 recipient	JMSN (total n = 160) beetle TC[2]	JMSN (total n = 12) filter TC
Resistance phenotype				
AmApFTAxCSSuTe	+			
NR		+		
AmCSSuTeNR			+(n = 141) 88%	+(n = 12) 100%
PCR-based replicon typing				
A/C, N	+			
FIA, FIB, FIC		+		
FIA, FIB, FIC, N			+(n = 152) 95%	

[1]+ = positive for the resistance phenotype.

[2]TC = transconjugants.

flocks as well as the dissemination of pathogens. There are many aspects to consider in eliminating or minimizing pathogens in the poultry house environment. In general, pathogens cannot be eliminated unless all reservoirs that harbor these agents are also eliminated. Insect vectors that serve as reservoirs for infectious agents are particularly troublesome. Not surprisingly, a variety of insects, such as crickets, grasshoppers, cockroaches, and beetles, have been found to harbor large bacterial concentrations (10^8 to 10^{11} per mL of gut) in their gut contents (Cazemier et al., 1997).

Bacteria are capable of rapid adaptation to their environment and 1 mechanism of adaptation is the exchange of mobile DNA elements such as plasmids, transposons, and integrons that carry genes necessary for survival. Such horizontal exchange has been shown to occur between pathogens and commensal bacteria in the gut of poultry (Poppe et al., 2005, 2006). The objective of this study was to determine if plasmid transfer could occur between bacteria within the confines of the gastrointestinal tract of lesser mealworm beetles that reside in poultry litter.

Surface disinfection of the beetles was done to assure that no donor or recipient bacteria were present externally. The experimental donor or recipient strains only grew on MacConkey agar with antibiotics to which they were resistant, as expected. There were tetracycline-resistant gram-negative bacteria on MAC-T plates within groups 1 and 2. However, further characterization determined these bacteria to be normal flora from the beetles, which was expected.

All of the beetles that fed on both donor and recipient bacteria produced transconjugants. The PBRT analysis of 160 selected transconjugants showed that all but one was positive for the N replicon. This replicon has been identified on a conjugative plasmid that was known to be possessed by the SN11 donor strain. The A/C replicon also present in the donor strain was nonconjugative in this study. The consistency of single plasmid transfer in 99% of the replicate beetle conjugations suggests that the beetle gut is an efficient in vivo model for natural conjugative transfer of large resistance plasmids.

The bla_{CMY} gene present on the A/C plasmid backbone confers resistance to the extended-spectrum cephalosporins (Winokur et al., 2001; Carattoli et al., 2002; Giles et al., 2004). Because the bla_{CMY} gene was not transferred and cephalosporins were not used for selection, it was not surprising that most of the transconjugants lacked resistance to the cephalosporins. These findings suggest that genes conferring resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline were present on the N replicon.

Horizontal transfer of the N plasmid replicon occurred at a high frequency in the beetle gut under the conditions used in this study. The small surface area of the beetle gut may have provided an optimal environment for successful conjugation. However, the amount of bacteria each beetle consumed and the degree of growth in the gut of donor and recipient strains during the overnight incubation was unknown; thus, it is difficult to directly compare the conjugation frequencies obtained from the beetle to those from the filters.

Studies of plasmid-mediated gene transfer in the digestive tract of silkworm larvae using the plant-epiphytic bacteria *Erwinia herbicola* have shown high conjugation efficiencies at 10^{-1} to 10^{-3} per recipient (Watanabe et al., 1998; Watanabe and Sato, 1998). However, no methodology described surface disinfection of the insect between the time of exposure to the donor and recipient bacteria. It is possible that plasmid transfer occurred externally and that the transconjugant was then ingested.

Petridis et al. (2006) force-fed house fly (*Musca domestica* L.) donor and recipient *E. coli* strains, thus eliminating the need for surface disinfection and allowing calculation of an exact treatment dose. They reported conjugation efficiency of 10^{-2} to 10^{-3} per donor cell in the gut 1 h after feeding. However, quantitation of transconjugant colonies was done using general-purpose Luria-Bertani media with appropriate antimicrobials. No further identification of transconjugant colonies was performed and as a result, contamination by intrinsically resistant normal flora or horizontal transfer to other intrinsically resistant recipients could have been included in the final counts.

Antimicrobial resistance plasmid transfer from *E. coli* to *Yersinia pestis* has been demonstrated in the adult flea midgut at a frequency of 10^{-3} 3 d postinfection (Hinnebusch et al., 2002). Four weeks postinfection,

Table 3. Conjugation frequencies observed from the beetle gut and filter controls

		95% CI[2]	
Item	GM[1] conjugation frequency	Upper	Lower
Beetle studies			
Study 1 (n = 10)	8.60×10^{-3}	2.0×10^{-3}	-2.4×10^{-3}
Study 2 (n = 14)	2.41×10^{-1}	5.3×10^{-1}	-5.6×10^{-1}
Study 3 (n = 16)	7.12×10^{-2}	3.4×10^{-1}	-3.4×10^{-1}
Filter studies			
Study 1 (n = 3)	9.06×10^{-3}	1.3×10^{-2}	-5.0×10^{-3}
Study 2 (n = 3)	2.34×10^{-3}	1.5×10^{-3}	-8.0×10^{-4}
Study 3 (n = 3)	8.75×10^{-3}	9.7×10^{-4}	-7.9×10^{-3}

[1]GM = geometric mean.

[2]CI = confidence interval.

95% of the fleas carried an average of 10^3 transconjugants.

This study demonstrates that conjugation and horizontal transfer of antimicrobial resistance genes can occur within the lesser mealworm gut at a high conjugation frequency. These data also suggest that litter beetles may be an active reservoir for these resistant bacteria, and likely facilitate the dissemination of antimicrobial resistance plasmids, by enhancing bacterial exchange of mobile DNA elements, among bacteria found in poultry production facilities.

ACKNOWLEDGMENTS

We thank Michael Talbert, Hollie Hunter, and Brendan Fitzpatrick for excellent technical assistance and Reiley Street for his insightful discussions (all from USDA, Agricultural Research Service).

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